Bovine Corneal Opacity and Permeability Test Method

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III. BOVINE CORNEAL OPACITY AND PERMEABILITY TEST METHOD

1.0 BCOP TEST METHOD RATIONALE

1.1 Scientific Basis for the BCOP Test Method

1.1.1 Mechanistic Basis of the BCOP Test Method

This Section of the BRD discusses the mechanistic basis for current test methods (i.e., the *in vivo* rabbit eye test) and the BCOP test method that is proposed as the initial test in a battery of tests to evaluate the ocular irritancy of new substances. The use of viable corneal tissue provides similarity to the actual system of interest -- the human eye. Opacity is an important endpoint in both test methods (BCOP and the *in vivo* rabbit eye test) and the human eye, although the BCOP test system as outlined in the proposed protocol does not allow one to differentiate the mechanistic cause of the corneal opacity. The BRD mentions only one mechanism of corneal opacity, but it is recognized that opacity can occur either because of severe injury, possibly with protein denaturation of the epithelial layer, or by swelling of the epithelium and/or corneal stroma. The latter is usually due to loss of the barrier function of the epithelial layer. Histopathological examination of the cornea will provide information useful to identify these mechanisms. Permeability is a measure of the integrity of the corneal epithelium and adds important information on the degree of injury that would be predicted by the test.

1.1.2 Advantages and Limitations of Mechanisms/Modes of Action of the BCOP Test Method

The BCOP method differs from the *in vivo* method in that it only evaluates the potential of a test material to damage the cornea of the eye. Some materials can cause serious corneal injury without appearing to change opacity or permeability immediately. For instance, cell death (e.g., apoptosis, necrosis) can selectively be induced by some chemicals (such as mustard gas), and such death may take place in keratocytes and vascular endothelium. Previous Expert Panels have suggested that methods to determine the irritation potential of test materials via the ocular route need to consider both damage to the cornea and damage to the vasculature and stem cells that grow in to repair the cornea (Nussenblatt et al. 1998). These cells, which are located at the rim of the cornea within the sclera (Schermer et al. 1986), are not normally evaluated in either the *in vivo* or *in vitro* systems.

The BRD mentions that injury to the sclera is not assessed in the BCOP assay, but no information is presented on whether serious damage to the sclera, including the limbal stem cells, can occur without evidence of injury to the cornea. Maurer and Jester in their series of papers, which report on *in vivo* ocular irritation studies of 23 materials that caused minimal to severe eye irritation, did not identify any materials that injured limbal stem cells without causing histological changes elsewhere in the cornea (reviewed in Maurer et al. 2002). Agents such as mustard gas can produce this type of damage in humans. Damage to the remainder of the eye and/or systemic toxicity is not addressed by this assay.

1.1.3 Similarities and Differences of Mechanisms/Modes of Action and Target Tissues Between the BCOP Test Method and Humans and Rabbits

Rabbit and bovine corneas both differ from human cornea. It is not known how these differences affect the ability of either the rabbit or bovine cornea to predict the response in the human, but the use of the *in vivo* rabbit test has apparently protected human populations from serious injury for many years.

1.1.4 Mechanistic Similarities and Differences Between the BCOP Test Method, the *In Vivo* Rabbit Eye Test Method, and/or Human Chemically-Induced Eye Injuries

The BCOP BRD does not include a discussion of the results of the studies by Maurer and Jester (reviewed in Maurer et al. 2002) in which they followed, using sequential *in vivo* confocal microscopy, the progression of eye lesions within the same animal over time. This extensive work was done on groups of rabbits exposed to 23 substances including surfactants, acids, alcohols, aldehydes, alkalis, bleaches, an aromatic amine, and a ketone. In addition to the sequential confocal examination of each animal, histopathological evaluations and live/dead staining studies were also done to confirm the results. These studies showed that "regardless of the process leading to tissue damage, extent of initial injury is the principal, mechanistic factor determining the outcome of the ocular irritation" (Maurer et al. 2002). These studies support the use of short-term assays to evaluate the long-term outcome of test substance exposure and should be discussed in the BCOP BRD. In addition, in human medicine, Hughes' classification is used to grade the severity of chemical injuries and predict the outcome based on initial injury. The classification includes the extent of corneal opacity (cloudiness) as judged by the visibility of the iris details, and the extent of limbal ischemia (based on the circumference involved) (Nussenblatt et al. 1998). The Draize and *in vitro* tests do not specifically examine limbal changes (Hughes 1946; McCulley 1987). More recent work supports the proposition that limbal stem cell injury predicts serious eye damage (Tseng and Sun 1999).

The BCOP BRD does not include a discussion of how protective mechanisms affect the outcome of the *in vivo* studies. Protective mechanisms are extremely important and are built into *in vivo* testing, but are absent in *in vitro* testing. The protective mechanisms include tearing and reflex blinking due to the activation of sensory trigeminal pathways, which in humans is interpreted as pain. However, note that for some test substances (e.g., solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. If an irritant not only causes cell/tissue damage, but also "denervates" the ocular nerve (sensory), this will alter the dynamics leading to more severe damage. This issue is not well covered in the BCOP BRD. The BCOP test proposed does not mimic these mechanisms. Consideration of the buffering effect of tears may be relevant to the apparent overprediction of injury by the BCOP for very dilute acids and bases.

The BCOP BRD reviews the important physiological and anatomical differences between the human eye and the rabbit eye, but provides little information with which to compare the bovine eye, other than the thickness of the corneal epithelium.

1.2 Regulatory Rationale and Applicability

1.2.1 Similarities and Differences Between Endpoints Measured in the BCOP Test Method and the *In Vivo* Rabbit Eye Test Method

The endpoint of corneal opacity is measured in both the BCOP and *in vivo* methods. However, the BCOP test method does not measure changes in the iris and conjunctiva, and does not identify substances systemically toxic via ocular exposure. The BRD states the BCOP does not assess reversibility without including a discussion of the work mentioned above (i.e., Maurer et al. 2002; Tseng and Sun 1999) that supports the concept that the final outcome of an eye injury can be predicted by the extent of the initial injury.

The BCOP BRD explains the current regulatory methods, including the differences between the three scoring systems (i.e., EPA 1996, EU 2001, UN 2003). The BRD points out clearly that there are no data comparing the results in the *in vivo* rabbit test to similar human exposure, except for very mild substances. Human ocular irritancy studies are not routinely conducted, and when they are only substances intended for use in or around the human eye (e.g., contact lens solutions, cosmetic formulations) are evaluated (Bruner et al. 1998; Cater et al. 2004). Historical experience indicates the rabbit test has protected human populations using existing scoring systems of the Federal Hazardous Substances Act (FHSA), EPA, and the EU.

1.2.2 Suggestions Regarding Other Evidence that Might be Used in a Tiered Testing Strategy

In addition to data from the BCOP test method, all other data on the test substance should be considered in the hazard and risk assessment of eye exposure, including the systemic toxicity of the material, information on related chemicals, possibly a structure activity or structure property analysis, its physicochemical properties, and the results of dermal testing. As *in vitro* tests become available for specific endpoints, toxicologists in industry and government will need to rethink their testing strategies, as it is very unlikely that the *in vitro* tests will be able to replace the current animal tests on a one-for-one basis.

Based on the information presented in the BRD, the Panel believes a sufficient mechanistic basis for the BCOP test method has been established.

2.0 TEST METHOD PROTOCOL COMPONENTS

2.1 Description and Rationale of the Components for the Recommended BCOP Test Method Protocol

2.1.1 Materials, Equipment, and Supplies

The suggested protocol does provide a standard procedure for obtaining eyes. The optimum age range for cattle should be determined; however, until this is evaluated, eyes should be obtained from young adult animals of 18-48 months of age. The protocol states eyes should be collected in a suitable container in Hanks Balanced Salt Solution (HBSS) containing antibiotics, and the container then maintained on ice. Use of antibiotics is questioned since they are not effective at 4˚C and because of this there is no rationale for their use if the eyes are adequately refrigerated. Eyes can probably be stored longer than the five hours stated in the protocol, possibly up to 12

hours, but this needs to be confirmed by careful examination of the eyes prior to testing. The single most important criterion for acceptance of eyes for use in the assay should be the careful examination of the eyes prior to dissection of the cornea and subsequent examination of the corneal preparation just prior to testing.

Eyes from animals that are sick or weakened should not be used because of concerns about zoonotic diseases, including Bovine Spongiform Encephalopathy (BSE). Standard laboratory precautions to protect against zoonotic diseases, such as use of gloves and eye protection, should be followed.

The Panel does not agree that sterile water is the preferred solvent for preparing solutions and suspensions; 0.9% NaCl is preferred. If solutions are diluted with distilled water, a distilled water control also needs to be evaluated. Distilled water itself can cause corneal damage and with edge damage from the corneal crush from the blocks, distilled water will further break down the epithelial barrier and cause corneal edema, as well as edema along the crush edge. Osmolarity and pH of the test solutions should be measured and recorded.

The BCOP assay should be optimized to decide which materials are used to bathe the cornea. It may not be necessary to add Fetal Bovine Serum (FBS), or even use Minimum Essential Medium (MEM). Balanced salt solutions designed for ophthalmic use may be more appropriate and may decrease cost as well.

The holder/clamp referenced in the BCOP BRD protocol does not maintain the bovine cornea with its natural curvature. The bovine cornea is oval in shape and has a radius of curvature. However, the blocks described in the BCOP BRD (Section 2.0) to mount the cornea are flat with round holes (17 mm); thus, when the cornea is clamped, the cornea surface can wrinkle, resulting in a loss of both epithelial and endothelial cells. Also, when the epithelium and endothelium wrinkle, there is loss of the corneal barrier function. The cornea needs to be mounted by clamping the sclera and the block needs to be designed with a radius of curvature appropriate for the bovine cornea.

Clamping directly on the cornea as described in the protocol leads to crush injury of the cornea. The crush zone, as well as the treatment area, are clearly seen in the picture on page 6 of the public comment letter dated November 18, 2004, from Drs. Harbell and Curren of the Institute for *In Vitro* Sciences (IIVS). The crushed area (edge damage) may have as much surface area as the treatment area. With edge damage, permeability of the sodium fluorescein will increase and the corneal response may be more severe as well as more variable. The use of the improved holder may also allow detection of limbal changes.

The papers by Ubels et al. (2002, 2004) referenced in the BCOP BRD and submitted as public comments (letter dated December 16, 2004, from Dr. Ubels) provide a good design of a holder large enough to clamp on the sclera and with the appropriate dimensions to maintain the natural curvature of the cornea.

2.1.2 Dose-selection Procedures

The BRD states dose-selection procedures are not relevant for the BCOP. However, there is discussion of various ways of dosing the eyes and dilution of the test materials in other sections.

2.1.3 Endpoint(s) Measured

Histopathological examination must be included unless the substance is from a class of materials known to be accurately predicted using only opacity and permeability in the BCOP assay.

A basic grading system that stresses utility needs to be established for the histopathological evaluation.

2.1.4 Duration of Exposure

The duration of exposure needs to be standardized (10 minutes - 4 hours) for certain types of test materials. In several places, the BCOP BRD discusses the fact that 10-minute exposure times cause volatile solvents to be overclassified by this method, but the protocol does not recommend a 3-minute exposure for these materials. This should be resolved before the protocol is finalized for volatile solvents.

The problem of the irritant potential of solids also needs to be defined more carefully. The very long exposures used are problematic, but since the application of solids to the conjunctival sac in Draize test rabbits also seems to be non-real-world, it is necessary to optimize the exposure time to solids in the BCOP assay. Perhaps further consideration should be given to the exposure method described by Casterton et al. (1996) for solid materials. Until these areas are optimized, the protocol does not appear to be appropriate for alcohols, ketones, and solids.

2.1.5 Known Limits of Use

The BCOP BRD discusses various known limitations. Based on information presented below (**Section III - 2.7**), the protocol outlined in the BRD, even with the additions described, is not appropriate for alcohols, ketones, and solids.

2.1.6 Nature of the Response(s) Assessed

Histopathological examination must be added unless the test substance is from a class of materials known to be accurately predicted using only opacity and permeability in the BCOP assay.

A basic grading system that stresses utility needs to be established for the histopathological examination.

2.1.7 Appropriate Controls and the Basis for Their Selection

As discussed in the BRD, every time a BCOP assay is run, a concurrent positive and a negative control needs to be included. A list of benchmark controls for common classes of chemicals should be suggested. Consideration should be given to the choice of a positive control liquid that is not an alcohol. Identification of reference substances that are part of the performance standards developed for the validated test method must be added.

2.1.8 Acceptable Range of Control Responses

Historical values for each testing facility should be used to set an upper value for the negative control and the acceptable range of values for the positive control.

2.1.9 Nature of the Data to be Collected and the Methods Used for Data Collection

The discussion and evaluation in the BCOP BRD are appropriate.

2.1.10 Type of Media in Which Data are Stored

Storage of data should comply with current GLP guidelines.

2.1.11 Measures of Variability

The discussion and evaluation are appropriate in the BCOP BRD.

2.1.12 Statistical or Nonstatistical Methods Used to Analyze the Resulting Data

The discussion and evaluation are appropriate in the BCOP BRD.

2.1.13 Decision Criteria and the Basis for the Algorithm Used

Because the BCOP test method proposed by the BRD is specifically for identification of ocular corrosives or severe irritants, the use of the calculated endpoint score and its cutoff point (i.e., decision criteria) should be re-examined. It may be that in comparison with the GHS classification system, examination of the individual scores or a different cutoff point for the calculated score would improve the accuracy and/or reduce the variability of the test. Finally, the use of the permeability endpoint only for some surfactants, but not all, is problematic. It may be that all surfactants should be evaluated using at least permeability and histopathology (as appropriate).

2.1.14 Information and Data That Will be Included in the Study Report

The opacitometer and corneal holder need to be carefully described in the test report.

2.2 Basis for Selection of the Test Method System

The discussion and evaluation in the BCOP BRD are appropriate.

2.3 Identification of Proprietary Components

The corneal holder should be carefully described in the protocol. Specifications for the type and use of the opacitometer should also be included in the protocol.

2.4 Numbers of Replicate and/or Repeat Experiments for Each Test

The discussion and evaluation are appropriate in the BCOP BRD.

2.5 Study Acceptance Criteria for BCOP Test Method

The discussion and evaluation in the BCOP BRD are appropriate.

2.6 Basis for any Modifications made to the Original BCOP Test Method Protocol

The discussion in the BCOP BRD is appropriate and the bases for the modifications are described adequately.

2.7 Adequacy of the Recommended Standardized Protocol Components for the BCOP Test Method

Solutions should be diluted in 0.9% NaCl whenever possible rather than in distilled water. With edge damage from the corneal crush from the holders, distilled water will further break down the epithelial barrier and cause corneal edema as well as edema along the crush edge. Distilled water itself can cause corneal damage. If solutions are diluted with distilled water, a distilled water control also needs to be evaluated.

The osmolarity and pH of test solutions should be measured and recorded. Solutions with osmolarity above 1000 are known to damage corneal epithelium.

Histopathological examination should be added to the recommended test protocol unless the test substance is known to be accurately predicted using only opacity and permeability.

Rinsing procedures should be optimized as a future improvement, particularly for viscous substances and solids.

With the addition of histopathology, the protocol as described in the BCOP BRD is appropriate for test materials other than alcohols, ketones and solids for the identification of corrosives and severe irritants in the test scheme described in the BRD. The Panel believes the other proposed changes could improve the test by reducing its variability and should be investigated as part of a continuing effort to improve the test.

3.0 SUBSTANCES USED FOR PREVIOUS VALIDATION STUDIES OF THE BCOP TEST METHOD

3.1 Substances/Products Used for Prior Validation Studies of the BCOP Test Method

Of the eight validation studies, three (Balls et al. 1995; Gautheron et al. 1994; Casterton et al. 1996) employed a broad range of chemical classes and products, and are considered adequate.

A total of 166 substances and formulations were evaluated in the eight studies. While the number of substances is considered adequate in the validation studies, methodological differences exist among these studies.

The Panel has encountered in human clinical practice materials that can cause severe eye damage without corneal opacity (Tseng S, personal communication). The Panel would like to be sure that representative types of these materials (e.g., heavy duty cleaning products for oven cleaning and drain cleaners) have been included in the prior validation studies. Materials known to be

severe eye irritants in humans, if they have not already been evaluated in the BCOP assay, should be tested in the assay.

Better characterization of physicochemical data on all the test substances is needed.

3.2 Coding Procedures Used in the Validation Studies

Coding is important; if it is not used, it may affect the data quality. Without coding procedures, concern may be raised regarding potential bias and quality of the *in vitro* test data. Except for one study (Casterton et al., 1996), the other studies appeared to employ coded substances. The coding procedures for these studies were considered adequate.

In summary, the data reviewed from prior validation studies in the BCOP BRD are considered adequate.

4.0 *IN VIVO* REFERENCE DATA USED FOR AN ASSESSMENT OF TEST METHOD ACCURACY

This section of the BCOP BRD provided a detailed analysis of the published *in vivo* methods used to evaluate ocular irritancy and/or corrosivity. The regulatory schemes for interpreting such *in vivo* data were provided in detail.

4.1 *In Vivo* Rabbit Eye Test Method Protocol(s) Used to Generate Reference Data

The *in vivo* rabbit eye test method protocol(s) used to generate reference data in the cited studies were appropriate.

4.2 Interpretation of the Results of the *In Vivo* Rabbit Eye Tests

The interpretation of the results of the *in vivo* rabbit eye tests was according to the EPA (1996), EU (2001), and GHS (UN 2003) classification systems. These systems as described have been judged by the agencies using these methods as suitable for their regulatory needs. The concern can reasonably be raised that these regulatory classification methods may not be adequate for use in evaluating or making distinctions between *in vitro* methods and their suitability for chemical or product class evaluations. In addition to the analyses conducted in the BCOP BRD, the Panel suggests an assessment based on ranking of experimental data for severity for both the reference method and the *in vitro* test.

4.3 *In Vivo* Rabbit Eye Test Data Quality with Respect to Availability of Original Study Records

In the case of the BCOP BRD, original study records, such as laboratory notebooks and raw data entry sheets, were not obtained for any of the reports evaluated. However, a lack of original study records does not necessarily raise concerns about a study. As long as an evaluation of the results can be made and the quality of the study otherwise is adequate (as is the case for the studies evaluated in the BCOP BRD), the study should be used.

4.4 *In Vivo* Rabbit Eye Test Data Quality with Respect to GLP Compliance

As far as the *in vivo* studies used for the accuracy analyses in Section 6.0 of the BCOP BRD, Balls et al. (1995) and Southee (1998) explicitly state GLP guidelines were followed. For the Bailey et al. (2004) report, about half of the *in vivo* studies were conducted according to GLP guidelines; for the other half, GLP compliance was not explicitly stated. For Gautheron et al. (1994), the *in vivo* studies were conducted according to European Economic Community (EEC) 1984 and 1991 test guidelines (predecessors of the current EU test guideline for eye irritation), but this information alone does not give enough information about GLP compliance. For the remaining reports (Swanson et al. 1995; Gettings et al. 1996; Casterton et al. 1996; Swanson and Harbell 2000), the extent of GLP compliance was not provided, so the extent of GLP compliance is not known.

4.5 Availability of Relevant Human Ocular Toxicity Information

ICCVAM should make an effort to obtain and consider information on human topical ocular chemical injury. It would seem worthwhile to determine if the current ocular hazard classification schemes are working correctly to protect workers and the public from severe eye injury by examining the injury databases maintained by the Poison Control Centers and the Department of Labor. The United States Eye Injury Registry (USEIR) may be another source of such information.

4.6 Accuracy and Reliability of the *In Vivo* Rabbit Eye Test

There should be more discussion of the variability of the rabbit data. This is particularly important in the determination of the accuracy of an *in vitro* test method. While there are often multiple results for each *in vitro* determination of irritation potential, there is only one *in vivo* result. Because of the known variability in the rabbit test, it is not possible from the data presented to determine if the inconsistencies between the two tests are due to "failure" of the *in vitro* test method or a misclassification by the single *in vivo* result provided. Historical data show that between 10% and 15% of the time a single rabbit test will misclassify a compound (Weil and Scala 1971; Kaneko 1996; Ohno et al. 1999). If this is the case, then 10% of the *in vivo* results are misclassified. Unfortunately, there is no way to determine which results are correct and which are not. An effort to determine if the *in vivo* results are consistent with the known toxicity of these materials would be useful (e.g., as indicated in the Registry of Toxic Effects of Chemical Substances [RTECS] or the International Uniform Chemical Information Database [IUCLID] databases).

However, data on the reproducibility or reliability of the *in vivo* rabbit eye test do exist in the literature, most notably the intra- and inter-laboratory study published by Weil and Scala (1971), as well as Kaneko (1996) and Ohno et al. (1999). Using a fixed protocol and a single supply of chemical agents tested in 25 laboratories, Weil and Scala (1971) identified "good" laboratories as those which had the lowest variance in ranking of irritancy using a sum of ranks statistical measure. They also found that nonirritants provided little useful information on laboratory performance. GLP regulations were not in place at the time of this study, but are not thought to

be critical in the evaluation of the data. The data from all three papers should be discussed in the BRD.

It is well documented that the Draize eye test has a very low variability at both ends of the MAS scale (e.g., the low end in the range of nonirritating chemicals and at the upper end of the scale in the range of severely irritating materials). However, in the middle range, the variability is very high (as indicated by the high CV and SD values in Balls et al. 1995, and Ohno et al. 1999).

When interpreting the *in vitro* test data, the differences in reproducibility/variability of the *in vivo* Draize eye test data have to be taken into account. Therefore, it has to be defined before data analysis is performed how this feature of the Draize eye test will be taken into account, when comparing it to results from *in vitro* tests and when attempting to determine the predictive value of the *in vitro* alternatives.

This important aspect has been cited as the main reason why the replacement of the Draize eye test by *in vitro* tests has failed in the past. As this view is well documented in the scientific literature (e.g., Balls et al. 1995), additional discussion in the BRD is warranted.

In summary, although the Panel believes there should be more consideration of the variability of the Draize data, the data are considered useful for evaluation of the BCOP assay.

Minority Opinion
This section was approved by consensus of the Panel with a minority opinion from Dr. Martin Stephens that sufficient animal data are available for further optimization/validation studies and no further animal testing should be conducted (See Minority Opinion from Dr. Stephens in **Section III - 12.3**).

5.0 BCOP TEST METHOD DATA AND RESULTS

5.1 BCOP Test Method Protocols Used to Generate Data Considered in the BRD

The Panel agrees with the BRD assessment of these data

5.2 Comparative BCOP Test Method–*In Vivo* Rabbit Eye Test Data Not Considered in the BRD

The Panel is not aware of other data that include the raw scores for both tests.

5.3 Statistical and Nonstatistical Approaches Used to Evaluate BCOP Data in the BRD

Within the context laid out in the ICCVAM Submission Guidelines (ICCVAM 2003), the statistical methods used to assess the data seem appropriate for these complex endpoints and provide a firm basis for further considerations across these data sets (BCOP BRD Sections 6.0 and 7.0). The conclusions relating to test method reliability (BRD Section 7.4) drawn from the analyses in BRD Section 7.0 seem sound.

5.4 Use of Coded Substances, Blinded Studies, and Adherence to GLP Guidelines

The Panel agrees with the BRD assessment of these data. The lack of GLP compliance should not *a priori* exclude data from evaluation.

5.5 "Lot-to-Lot" Consistency of the Test Substances and Time Frame of the Various Studies

The Panel agrees with the BRD assessment of these data. However, many of the substances used in the accuracy and reliability calculations are classified in Appendix E of the BCOP BRD not as 'liquid' or 'solid' but instead as 'not provided'. Since one of the issues for the BCOP is the problem with solids, it would be helpful to obtain physicochemical information on as many of these materials as possible. The use of 'volatile solvents' is described in the BRD as problematic with the 10-minute exposure time. The Panel evaluation of the data indicates that alcohols and ketones are the problematic substances, but additional physicochemical data are needed to refine this evaluation.

In summary, the *in vitro* data are sufficient and acceptable, but more data on the physicochemical characteristics of the test substances are needed.

6.0 BCOP TEST METHOD ACCURACY

6.1 Accuracy Evaluation of the BCOP Test Method for Identifying Ocular Corrosives and Severe

The accuracy of the BCOP test method has been evaluated in comparison to the EPA (1996), EU (2001), and the GHS (UN 2003) ocular irritancy classification systems assuming the formula used to calculate the *in vitro* score currently used is optimal for identifying severe irritants. The discussion is very complete and the data are presented clearly.

Because the Panel does not have data that could give information on the variability in the *in vivo* test results, it is difficult to determine if the single rabbit test being used as the "reference standard" is in fact an "accurate" rabbit test. Combining all *in vitro* results on a substance into a single value minimizes the variability of the data and appears to be the best approach for obtaining an accurate *in vitro* number, realizing the variability has been defined during the inter- and intra-laboratory comparisons. However, without similar information on the accuracy of the *in vivo* results, statistical comparisons are very one sided. As discussed previously, it can be assumed from past experience that 10% to 15% of the *in vivo* results from a single assay are 'wrong' (Weil and Scala 1971; Kaneko 1996; Ohno et al. 1999). The Panel is aware that NICEATM conducted an analysis of the variability of the *in vivo* test method and believes the final decision on what can be said about accuracy should be made after reviewing the results of the NICEATM study. In addition, the Panel recommends scanning other publicly available sources of eye irritation data (e.g., RTECS or IUCLID databases) to determine if the *in vivo* data used in these studies is comparable to the results now accepted for regulatory purposes.

The Panel has been asked to compare the data to three different regulatory standards. There are two sources of variability when comparing these results. First, the rabbit tests were evaluated in different ways and, secondly, different lists of substances could be evaluated for different regulatory standards. It is not clear if the Panel should suggest the use of the BCOP test method for one regulatory agency scheme but not another.

In addition, the use of single numbers for the various accuracy calculations is misleading. This approach gives the appearance that the *in vivo* tests used for comparison are 100% accurate and there is no possible source of variability around these numbers. The numbers should be clearly presented as concordances with a single Draize test result.

The Panel would like to point out that the scientific justification for the classification schemes for the *in vivo* data is not being examined in this review and this could well be a significant source of both variability in the *in vivo* test and the apparent lack of accuracy in the *in vitro* test as compared to the three regulatory classification schemes. This is particularly true for the two schemes that at least in part base their classification on the result of a single rabbit (i.e., EPA 1996; UN 2003), which would appear to increase the possibility of test-to-test variability as shown by Kaneko (1996), and for which there are no data on the variability of the *in vivo* results.

Minority Opinion
Drs. Martin Stephens and Peter Theran note that the term "accuracy" is used throughout the four BRDs and this Panel Report to address the degree of consistency between the *in vivo* rabbit (Draize) test and each of the four *in vitro* alternative test methods being evaluated.

It is well documented that there is a significant degree of variability in the data produced by the *in vivo* rabbit eye test when it is compared with itself, which raises the question as to the accuracy of the *in vivo* test to predict the human experience. Given this variability and the fact that no data demonstrating the ability of the *in vivo* test to predict the human experience was presented to the Panel, Drs. Stephens and Theran feel it should be recognized that this test is an imperfect standard against which the new tests are being measured.

Drs. Stephens and Theran are filing a minority report because they believe that the term "accuracy" is inappropriately used, and that it is more appropriate to use the term "consistency with *in vivo* data" when comparing test results.

6.2 Strengths and Limitations of the BCOP Test Method

The strengths and limitations identified within the confines of the substances tested are adequately discussed in the BCOP BRD with the exception of the effect of colored substances. Again, this determination is hampered by the lack of similar data obtained using the *in vivo* protocol. The exploration of the effects of physicochemical properties is limited. In the future, consideration should be given to exploring these effects further using a structure activity or structure property relationship program.

6.3 BCOP Test Method Data Interpretation

Issues of test data interpretation have been adequately addressed in the BCOP BRD. In addition to the analyses conducted, the Panel suggests an assessment based on ranking of experimental data for severity for both the reference method and the *in vitro* test.

In summary, the test method is accurate for identification of corrosive and severely irritating substances, except for alcohols, ketones, and solids, when used in the tiered testing scheme described in the BCOP BRD.

7.0 BCOP TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

7.1 Selection Rationale for the Substances Used in the BCOP Test Method Reliability Assessment

The Panel agrees with the BRD assessment of these data.

7.2 Intralaboratory Repeatability and Intra- and Inter-laboratory Reproducibility of the BCOP Test Method

The BCOP BRD concludes, in Section 7.4, that while the intralaboratory repeatability and the intra- and inter-laboratory reproducibility of the BCOP test method appear sufficient for its general application to the detection of ocular corrosives and severe irritants, further work may be needed to reduce interlaboratory variability associated with alcohols, organic solvents and solids. After reviewing the data, the Panel agrees the intra- and inter-laboratory reproducibility of the test appear sufficient and that alcohols and solids need to be reviewed. From the data provided it is difficult to determine if it is organic solvents in general that are a problem. The data provided indicate that ketones also need to be reviewed.

CV values should be used with care with this data because the scores can range from 200 to less than 1. The median and mean CV data may not be informative because it will depend greatly on the scores of the individual tests used in the analysis; that is, comparing the means of the CVs of a set of results with predominantly high scores with a set of results with predominantly low scores is inappropriate.

The data from existing studies have been extensively reviewed and considered in the BCOP BRD. The impression from the summary and conclusions is that the test method showed acceptable levels of intralaboratory repeatability and reproducibility, and interlaboratory reproducibility. Note, though, that in Southee's interlaboratory comparison (Appendix F of the BCOP BRD), there are highly significant differences between the three laboratories in the values they obtained for the *in vitro* scores for ethanol, although variability between and within experiments in the same laboratory was low. The mean score for the three laboratories was 46.3 (SD = 9.7; CV = 21%). This indicates that even with good laboratories, a standard protocol, and a "simple" substance, significant differences in response can occur. It also supports the comment in the summary that further work may be needed to reduce interlaboratory variability.

7.3 Availability of Historical Control Data

The Panel agrees with the BRD assessment of these data.

7.4 Effect of Minor Protocol Changes on Transferability of the BCOP Test Method

The test method proposed is robust. Several additions to the currently used protocol have been proposed in the BCOP BRD to standardize current practice. Further suggestions have been made by this Panel to reduce variability within and between laboratories. Whether adopting these suggestions will actually reduce variability will need to be determined experimentally.

In addition, many of the suggestions for the protocol seem to come from IIVS. This is a good laboratory with a lot of experience, so their suggestions are important. On the other hand, it would be useful to determine if other laboratories believe the changes that have been suggested are possible within their constraints.

In summary, the inter- and intra-laboratory reproducibility of the method is acceptable.

8.0 TEST METHOD DATA QUALITY

8.1 Impact of GLP Noncompliance and Lack of Coded Chemical Use

The quality of the data used in the BCOP BRD is adequately described. Failure to use coded substances or to follow GLP guidelines significantly impacts on the quality of some data presented in the BRD. Coding was not used for one study but this study was not utilized in the accuracy analysis using pooled data from different studies. Coding should be used for all subsequent studies.

8.2 Results of Data Quality Audits

The Panel agrees with the BRD assessment of these data. Spot checks of data not part of the multilaboratory validation studies could be conducted; however, the Panel does not believe this is necessary.

8.3 Impact of GLP Deviations Detected in the Data Quality Audits

The BRD assessment of these data is appropriate.

8.4 Availability of Original Records for an Independent Audit

The availability of notebooks is described in the BCOP BRD. The lack of original notebook data for this review is of some concern but not sufficient to remove the data from consideration. Information presented at the January 11-12, 2005, meeting indicates that raw data may be available for many, if not all, of the studies included in this evaluation. The ICCVAM recommendation that all data supporting validation of a test method be available with the

detailed protocol under which the data were produced is reasonable and should be supported (ICCVAM 1997, 2003).

In summary, the Panel believes the data quality is sufficient.

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Other Published or Unpublished Studies Conducted Using the BCOP Test Method

Relevant data appear to be identified. The BCOP test bears direct biological relevance to the Draize test.

9.2 Conclusions Published in Independent Peer-Reviewed Reports or Other Independent Scientific Reviews

The Panel agrees with the BRD assessment of these data.

9.3 Approaches to Expedite the Acquisition of Additional Data

NICEATM has made every attempt to obtain available data. It is possible that more data could be obtained by working through trade associations, but much of the data in the BCOP BRD comes from these sorts of efforts, so whether more data could be obtained is unclear.

In summary, the additional data have been adequately reviewed.

10.0 ANIMAL WELFARE CONSIDERATIONS (REFINEMENT, REDUCTION, AND REPLACEMENT)

10.1 Extent to Which the BCOP Test Method Refines, Reduces, or Replaces Animal Use

The BCOP BRD adequately addresses these issues. Use of the BCOP test method will result in the use of fewer animals by classifying some substances without further animal tests and reduce the number of animals exposed to severe irritants.

In summary, the BCOP BRD adequately addresses animal welfare considerations.

11.0 PRACTICAL CONSIDERATIONS

11.1 BCOP Test Method Transferability

11.1.1 Facilities and Major Fixed Equipment Needed to Conduct the BCOP Test Method
The BCOP BRD addresses these considerations adequately.

11.1.2 General Availability of Other Necessary Equipment and Supplies
The BCOP BRD addresses these considerations adequately.

11.2 BCOP Test Method Training

11.2.1 Required Training Needed to Conduct the BCOP Test Method
The BCOP BRD addresses these considerations adequately.

11.2.2 Training Requirements Needed to Demonstrate Proficiency
The BCOP BRD addresses these considerations adequately with the exception that the description of training of technicians for the *in vivo* test may be improper -- the technicians essentially have to demonstrate proficiency in the *in vivo* test the same way as in the *in vitro* test.

A training video and other visual media on the technical aspects of the assay are recommended. Training approaches in the application of this test method should be developed and implemented.

11.3 Relative Cost of the BCOP Test Method

The BCOP BRD addresses these considerations but the discussion should be modified to reflect the public comments submitted by S.C. Johnson & Son, Inc. in December 2004 on the costs and time comparisons with the Draize test.

11.4 Relative Time Needed to Conduct a Study Using the BCOP Test Method

For very corrosive substances and some severe irritants, the evaluation may be completed within four hours in the *in vivo* test, since animals should be killed for humane reasons if severe lesions are seen.

In summary, the Panel sees no serious practical issues with the use of the BCOP test method.

12.0 PROPOSED TEST METHOD RECOMMENDATIONS

12.1 Recommended Version of the BCOP Test Method

12.1.1 Most Appropriate Version of the BCOP Test Method for Use in a Tiered Testing Strategy to Detect Ocular Corrosives and Severe Irritants and/or for Optimization and Validation Studies
For the purpose of identifying corrosive or severe eye irritants in the tiered testing scheme outlined in the BRD, the proposed version of the BCOP test method has been shown to have adequate accuracy and reliability for detecting corrosive or severe eye irritants, with the exception of the caveats described in **Section III - 12.2** of this report.

12.2 Recommended Standardized BCOP Test Method Protocol

For the purpose of detecting severe eye irritants in the tiered testing scheme outlined in the BRD, the proposed BCOP test method protocol is useful for identification of severe or corrosive ocular irritants with the following caveats:

- The test should not be used to identify corrosive or severely irritating ketones, alcohols, and solids. Further optimization and validation are necessary before these classes of materials can be assessed with this test.
- It needs to be confirmed that the BCOP test method can identify, as well as or better than the Draize test, those substances known to cause serious eye injury in humans. It appears from the list of chemicals tested that at least some of these substances have been tested in BCOP (e.g., floor strippers, heavy duty cleaners).
- Users should be aware of zoonoses, including the possibility of BSE.
- A histopathological examination should be added to the test unless the test substance is from a class of materials known to be accurately predicted using only opacity and permeability in the BCOP assay.
- Concurrent negative, positive, and benchmark controls should be used.
- 0.9% NaCl should be used instead of distilled water as the test substance diluent.
- Determination of osmolarity and pH of test solutions should be conducted.
- The optimum age range for cattle should be determined.

12.2.1 Appropriateness of the Recommended Standardized Test Method Protocol and Suggested Modifications to Improve Performance

The following are recommended as modifications that might improve the accuracy and reliability (repeatability/reproducibility) of the BCOP test method:

- Use of the larger holder as suggested by Ubels et al. (2002, 2004)
- Re-examine the use of the calculated total score when the endpoint is serious injury only
- Changes to the medium used to bathe the eyes including a determination of whether FBS is needed

While these modifications are important, the data presented in the BRD support use of the BCOP assay in its current form for identifying ocular corrosives and severe irritants other than alcohols, ketones, and solids in a tiered testing strategy for regulatory hazard classification and labeling purposes.

12.2.2 Other Endpoints that Should be Incorporated into the BCOP Test Method

Histopathological examination should be added to the recommended test protocol unless the test substance is from a class of materials known to be accurately predicted using only opacity and permeability in the BCOP assay.

While actually a change to the BCOP method, the Panel calls attention to the possibility that porcine eyes might also be a useful model for human eyes. This change would require complete validation, but the Panel wants to be sure this possibility is considered for future work.

Minority Opinion
Dr. Freeman expressed no opinion as to whether the BCOP assay had met the validation criteria as set forth in Appendix D of the ICCVAM Submission Guidelines (2003). This is because the question of whether these validation criteria had been met never reached a conclusive decision by the Panel. This is the basis for his abstention from voting on the acceptance of **Section III - 12.2**.

The Panel raised the question as to whether the BCOP assay could be considered validated. This was determined to not be a function of the Panel; however, it was also determined that it was a function of the Panel to judge whether the validation criteria (as set forth in the ICCVAM guidelines cited above) had been met. Although the Panel report on the BRD addressed the validation criteria, during the discussion, it seemed that some Panel members were unclear as to whether they had been asked to specifically answer this question in a summary manner. Thus, no summary conclusion was reached on whether the validation criteria were fulfilled, and under time constraints to end the Panel review on schedule, the adopted language was that the assay "was useful" in the identification of severe irritants or corrosives to the eye.

The discussion regarding BCOP could have been resolved more definitively with a few minor changes to the process, as noted below:

- The Panel should have been clearly instructed and reminded as necessary that it was to conclude whether the available information on the assay fulfilled the validation criteria.
- When it became clear that there was confusion on the ultimate objective, the tasking should have been clarified and possibly a recess called to permit appropriate deliberation. Please keep in mind the extensive preparatory work (and cost) prior to the Panel meeting.

It is suggested that a pro forma checklist be developed as an aid to guide future Expert Panels to final resolution of their assigned tasks, e.g., determining the validation status, that is, whether validation criteria, have been met.

Minority Opinion
Drs. Theran and Stephens state that the chair of the BCOP group summarized the group's findings and conclusions on the afternoon of January 12th, during the plenary, public session of the full expert panel. The group's key conclusion was that the BCOP had satisfied ICCVAM's validation criteria, and therefore the validation status of the BCOP test method should be characterized as "valid" for the purpose of serving as a positive screen for severe or corrosive eye irritants. The BCOP group chair noted that as with all methods previously shown to be valid by ICCVAM, ECVAM, and others, the BCOP test method has particular strengths and limitations that should be taken into account when the method is used.

Drs. Theran and Stephens object to the pressure brought to bear on the BCOP group that ultimately led the members, under duress, to withdraw their summary conclusion that the test method was valid and to substitute the tepid and vague language from other group reports that the test method was "useful." They believe that ICCVAM personnel and panel members were incorrect in stating that the charge to the four groups did not include drawing conclusions about

the validation status of the test methods under review. The very title of the 18-page charge to the panel was "Guidance to the Expert Panel for Evaluation of the Validation Status of the BCOP, ICE, IRE, and HET-CAM Test Methods for Identifying Ocular Corrosives and Severe Irritants" (emphasis added). After much heated discussion, the BCOP group was given the opportunity to make a statement on the validation status of the BCOP method, but the group had been subjected to such counter pressure by that point that they understandably decided against characterizing the method as valid.

An official effort to clarify the charge to the group on the final morning of our 4-day effort was helpful, but once again lead to heated discussion that muddied the waters.

This minority opinion was filed because Drs. Theran and Stephens believe the BCOP group was inappropriately pressured to withdraw its main scientific finding. The final report should have concluded that the BCOP has been found to be valid, within the identified limits, and that any further optimization or other studies should not be cause for delaying regulatory agency review for test method acceptance.

12.3 Recommended Optimization and Validation Studies

12.3.1 Recommended Optimization Studies to Improve Performance of the Recommended BCOP Test Method Protocol

Future improvements to improve the accuracy and reliability (repeatability/reproducibility) are recommended including use of the larger holder similar to that suggested by Ubels et al. (2002), re-examining the use of the calculated total score when the endpoint is serious injury only, changes to the medium used to bathe the eyes, avoiding use of antibiotics, and appropriate ages of donor animals. While these improvements are important, the data presented in the BRD are sufficient for supporting use of the BCOP assay in identifying ocular corrosives and severe irritants, except for alcohols, ketones and solids, in a tiered testing strategy for regulatory hazard classification and labeling purposes.

The optimization study design recommended in the BCOP BRD is appropriate.

12.3.2 Recommended Validation Studies to Evaluate Performance of the Optimized BCOP Test Method Protocol

Validation studies, or submission of additional data supporting the three-minute exposure time suggested for volatile solvents, will be necessary before the BCOP test method can be recommended for use with alcohols and ketones. Validation studies or submission of additional data will be necessary before the BCOP test method is acceptable for solids.

The information in the BCOP BRD, along with the additions of our suggestions, is sufficient to support the use of this test method to identify severe irritants and corrosives, with the exception of alcohols, ketones, and solids, in the tiered testing scheme described in the BRD.

It is understood that adding histopathological examination to the test method involves additional endpoints, but current practice has not been to insist on validation of histopathological examination when it is added to an *in vivo* test method. Thus, there is no need for an additional

validation study based solely on the addition of this endpoint. A standardized histopathological scoring system is suggested, but this should be arrived at by the experts in the field and will not require validation. NICEATM/ICCVAM should facilitate the development of a histopathological scoring system for corneal damage (with visual aids).

Changes in the calculation method for the BCOP test score, or the use of the individual endpoint data instead of a calculated score also do not need to be validated.

When validation studies are conducted, the studies proposed in the BCOP BRD are appropriate but should be limited to the classes of test substances in question. Validation studies should be carefully planned. Tests should first be done to confirm that any modifications of the protocol do not decrease reliability. Once the inter- and intra-laboratory variability is defined, it will not be necessary to have a large number of laboratories test every chemical in the validation study. Validation should focus on the class of chemicals in question. The study should involve a very small number of experienced laboratories with only a limited number of duplicate samples at each laboratory.

Any validation or optimization studies should use existing animal data, if available. Additional animal studies should only be conducted if important data gaps are identified and such studies should be carefully designed to maximize the amount of pathophysiological information obtained (e.g., wound healing) and to minimize the number of animals used.

Minority Opinion

According to Dr. Martin Stephens, **Section III – 12.3** recommends that additional optimization and/or validation studies be conducted, and the report leaves open the possibility of additional animal studies as part of this process. Dr. Stephens believes that no additional animal studies should be conducted for such optimization or validation exercises. He cited several reasons for holding this view:

1. Draize testing of severely irritating or corrosive chemicals causes extremely high levels of animal suffering.
2. The intended purpose of the alternatives under review is narrow in scope (i.e., simply to serve as a positive screen for severely irritating or corrosive chemicals). Negative chemicals go on to be tested in animals.
3. The Panel learned that more animal and alternative data exist that are relevant to each of the alternative methods, and greater efforts should be made to procure these and any other existing data.
4. Some relevant animal data were dismissed from the analysis of each alternative method, and this dismissal should be reevaluated in light of any need for additional data.
5. Suggestions for further optimization and/or validation studies should be assessed critically, in light of the fact that only the most promising alternative method need be developed further, not necessarily all four methods, and that whatever alternative is selected for further development need be optimized only to the point at which it is at least as good as the Draize test.

6. A new modular approach to validation has been developed that could potentially reduce the number of chemicals needed to fulfill each module. Such an approach, if pursued, might be workable with the data already summarized in the BRDs.

12.4 Proposed Reference Substances for Validation Studies

See **Section V**.

13.0 BCOP BRD REFERENCES

13.1 Relevant Publications Referenced in the BRD and any Additional References that Should Be Included

The papers of J.V. Jester and J.K. Maurer should be added as they support the use of short-term endpoints to predict longer-term results.

Also add to the BCOP BRD any other publications cited in **Section III** of this report and listed below that were not included in the BRD.

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